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TECHNICAL MANUSCRIPT 518

SPIRACULAR RESPONSES
OF Aedes MOSQUITOES
TO CARBON DIOXIDE AND OXYGEN

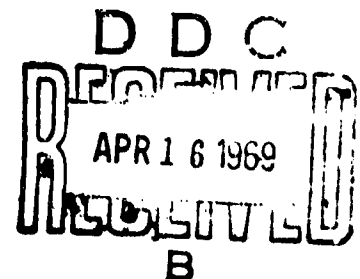
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Elliot S. Krafur
Charles L. Graham

MARCH 1969

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TO CARBON DIOXIDE AND OXYGEN

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PLANT SCIENCES LABORATORIES

Project 1B562602A059

March 1969

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ABSTRACT

Observations were made on the responses of the thoracic spiracles of Aedes aegypti and Aedes triseriatus to a constant carbon dioxide pressure in serial concentrations of oxygen in nitrogen and to the oxygen in nitrogen series alone. Lower than atmospheric pressures of oxygen caused an increase in the frequency and duration of spiracular opening. Higher than atmospheric pressures of oxygen had no effect on frequency of spiracular opening, but reduced the duration of opening. Carbon dioxide effected full spiracular opening in proportion to the oxygen concentration. The duration of spiracular opening, regardless of amplitude, was also proportional to the ratio of carbon dioxide to oxygen. Responses of Aedes spiracles to hypoxia and carbon dioxide decreased with increasing duration of exposure.

CONTENTS

Acknowledgments	2
Abstract	2
I. INTRODUCTION	5
II. METHODS	5
III. RESULTS	6
IV. DISCUSSION	14
Literature Cited	17
Distribution List	19
DD Form 1473	21

FIGURES

1. Frequency of Spiracular Opening and Total Time of Opening in <u>A. triseriatus</u> as Functions of O ₂ Concentration	8
2. Amplitude of Spiracular Opening in <u>A. triseriatus</u> as a Function of the CO ₂ /O ₂ Ratio	10
3. Spiracular Responses of <u>A. aegypti</u> and <u>A. triseriatus</u> to 1% Carbon Dioxide in Air as a Function of Duration of Exposure . . .	12
4. Spiracular Responses of <u>A. aegypti</u> and <u>A. triseriatus</u> to 8% Oxygen as a Function of Duration of Exposure	13

TABLES

1. Spiracular Behavior of <u>A. triseriatus</u> Exposed to Atmospheres of 20% and 100% Oxygen with and without a Carbon Dioxide to Oxygen Ratio of 2.5%	6
2. Spiracular Responses in <u>A. triseriatus</u> (Say) to Varying Levels of Oxygen and Nitrogen with a Constant Level of Carbon Dioxide at 0.005% of the Total Atmosphere	9

I. INTRODUCTION*

Hazelhoff¹ in 1926 first reported that spiracles of the cockroach opened in response to carbon dioxide. Since then, many efforts have been made to define the mechanism and find the site of action of this gas.²⁻¹¹ Several investigators have noted that carbon dioxide - induced spiracular opening was proportional to the oxygen tension,^{2,7,9,11} and in fact graded spiracular opening was observed in locusts,⁸ cockroaches¹ and Musca and Callitroga adults² as a result of varying ratios of carbon dioxide to oxygen. In view of the dominant role these two gases play in control of spiracular opening and closing, it was considered desirable to measure their sensory effects in Aedes. Mosquitoes do not normally experience ambient concentrations of these gases as employed here. The assumption was made that the experimental gas regimes provided to the external environment would elicit the same spiracular responses as similar intratracheal gas tensions. Mosquitoes proved ideal subjects because their spiracles were easy to observe, exhibited graded opening, and did not become involved with possibly complicating ventilatory movements.

II. METHODS

The behavior of the thoracic spiracles was observed and recorded as previously outlined.¹² Gas mixtures were prepared from cylinders of oxygen, nitrogen, and carbon dioxide, and were measured with flow meters. With the exception of carbon dioxide, gases were bubbled through water to give a relative humidity of 85 to 95%. Carbon dioxide was used in small amounts and applied directly to the gas delivery tube. Spiracular opening and closing was noted as follows: Freq. refers to the number of openings per minute of observation, toD indicates the number of seconds per minute that spiracles were observed partially open, toF is the number of seconds per minute that spiracles were fully open, and toT is the total seconds per minute that spiracles were open regardless of amplitude. An account of the nature of thoracic spiracular opening is given in a previous report.¹²

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III. RESULTS

A preliminary experiment was designed to test the hypothesis that spiracular response to carbon dioxide was proportional to the oxygen tension. Spiracular behavior was compared among a group of A. triseriatus randomly exposed to atmospheres of the same proportions of the two gases but different absolute amounts of each. Control observations were made in atmospheres of 20% oxygen (air) or 100% oxygen (Table 1). As might be expected, spiracular opening was significantly more conservative in mosquitoes exposed to the higher oxygen tension ($P < 0.05$). This, however, was not reflected in amplitude of opening, but only in duration. In contrast, no differences in spiracular behavior were apparent in these same atmospheres when carbon dioxide was injected in similar ratios. Thus, both amplitude and duration of spiracular response to carbon dioxide was directly proportional to the oxygen tension.

TABLE 1. SPIRACULAR BEHAVIOR OF A. TRISERIATUS EXPOSED TO ATMOSPHERES OF 20% AND 100% OXYGEN WITH AND WITHOUT A CARBON DIOXIDE TO OXYGEN RATIO OF 2.5%

Treatment	Freq.	S.E.	toF	S.E.	toT	S.E.
N ^a / = 20 100% O ₂	2.75	±0.39	-	-	26.1 ^b /	±3.9
N = 20 20% O ₂	2.90	±0.54	-	-	34.8 ^a /	±4.7
CO ₂ + 100% O ₂	1.87	±0.24	42.5	±3.4	48.2	±2.3
CO ₂ + 20% O ₂	2.20	±0.36	40.9	±4.1	49.2	±2.3

a. N = number of observations.

b. $P < 0.05$.

Having demonstrated a sensory effect of oxygen and carbon dioxide on spiracular opening and a relationship of carbon dioxide - induced opening to oxygen tension, an attempt was made to define spiracular behavior as functions of (i) oxygen tension alone and (ii) a graded series of ratios of the two gases. Extensive observations were made on A. triseriatus mosquitoes exposed to a constant 25 cc/min carbon dioxide in atmospheres of 10, 15, 20, 30, 40, and 50% oxygen diluted with nitrogen or in 100% pure oxygen. Oxygen, rather than carbon dioxide, was varied because large volumes of gas could be more accurately delivered than very small amounts. Individual insects were observed first in gas flow without carbon dioxide for a 1-min observation period, and subsequently in oxygen and nitrogen plus carbon dioxide for the following 2 min. An accommodation period of 2 to 3 minutes in the chosen oxygen tension was

provided. The data therefore reflected spiracular behavior in seven oxygen tensions and seven ratios of carbon dioxide to oxygen. A total of three samples of ten mosquitoes each were randomly examined in each oxygen tension.

Spiracular responses to oxygen tensions of 10 to 100% in the absence of ambient carbon dioxide are illustrated in Figure 1. To examine frequency of spiracular opening first, it is clear that a logarithmic rate of decrease occurred with increasing oxygen concentration; this abruptly leveled off beyond 20% oxygen with no further significant change. Remarkably similar frequency responses to oxygen tensions above the 20% value occur in *Aedes*, which is in contrast to Wigglesworth's¹¹ finding in *Xenopsylla*, which showed that frequency of opening and closing continued to decrease in oxygen values greater than 20%. Expressed as total time of opening (toT), the average time that spiracles were open per minute of observation was inversely proportional to the oxygen tension. The average slope of the response was such that for each 10% increase in oxygen, a corresponding decrease of 2.6 seconds per minute was obtained in spiracular opening. The slope was greatest in the range of 20 to 30% oxygen, where a decrease of 9.2 seconds in spiracular opening was observed. Further 10% increases in oxygen concentration beyond the 50% level resulted in a slope of only 0.51 second. Amplitude of spiracular opening (toF) as a function of oxygen alone was of little importance because only twitch-like openings were observed in all but the lowest two oxygen concentrations. Thus the relationship of spiracular behavior to higher than normal ambient oxygen tensions is such that frequency was not immediately affected but duration of each opening decreased; in lower than normal ambient oxygen tensions both frequency and duration of opening were elevated.

Parameters representing spiracular behavior as functions of ratios of carbon dioxide and oxygen are shown in Table 2 and Figure 2. Comparison of the control values with those obtained in identical oxygen tensions plus carbon dioxide indicate that an average 11% increase in the mean time of spiracular opening (toT) occurred. However, the increase in toT was not constant for each oxygen tension employed. In the range of 10 to 20% oxygen, addition of carbon dioxide effected a mean 14% increase in spiracular opening; between 30 and 100% oxygen, an average 9% increase was observed. The average slope of spiracular response (toT) was a 2.6 seconds decrease per minute for each 10% increase in oxygen. This value is quite similar to that obtained in the control observations. Thus, the average duration of spiracular openings per minute (toT) were more closely related to oxygen tension than to the ratio of carbon dioxide to oxygen. On the other hand, values representing frequency of spiracular opening and amplitude of opening (toF) in oxygen plus carbon dioxide were not at all like those obtained in oxygen alone. As a function of increasing oxygen concentration (but decreasing per cent of carbon dioxide in relation to oxygen), frequency of spiracular opening increased up to the 40% oxygen level, or 1.25% carbon dioxide of oxygen concentration. Further increases in oxygen resulted in a decrease in frequency of opening. This decrease was insignificant

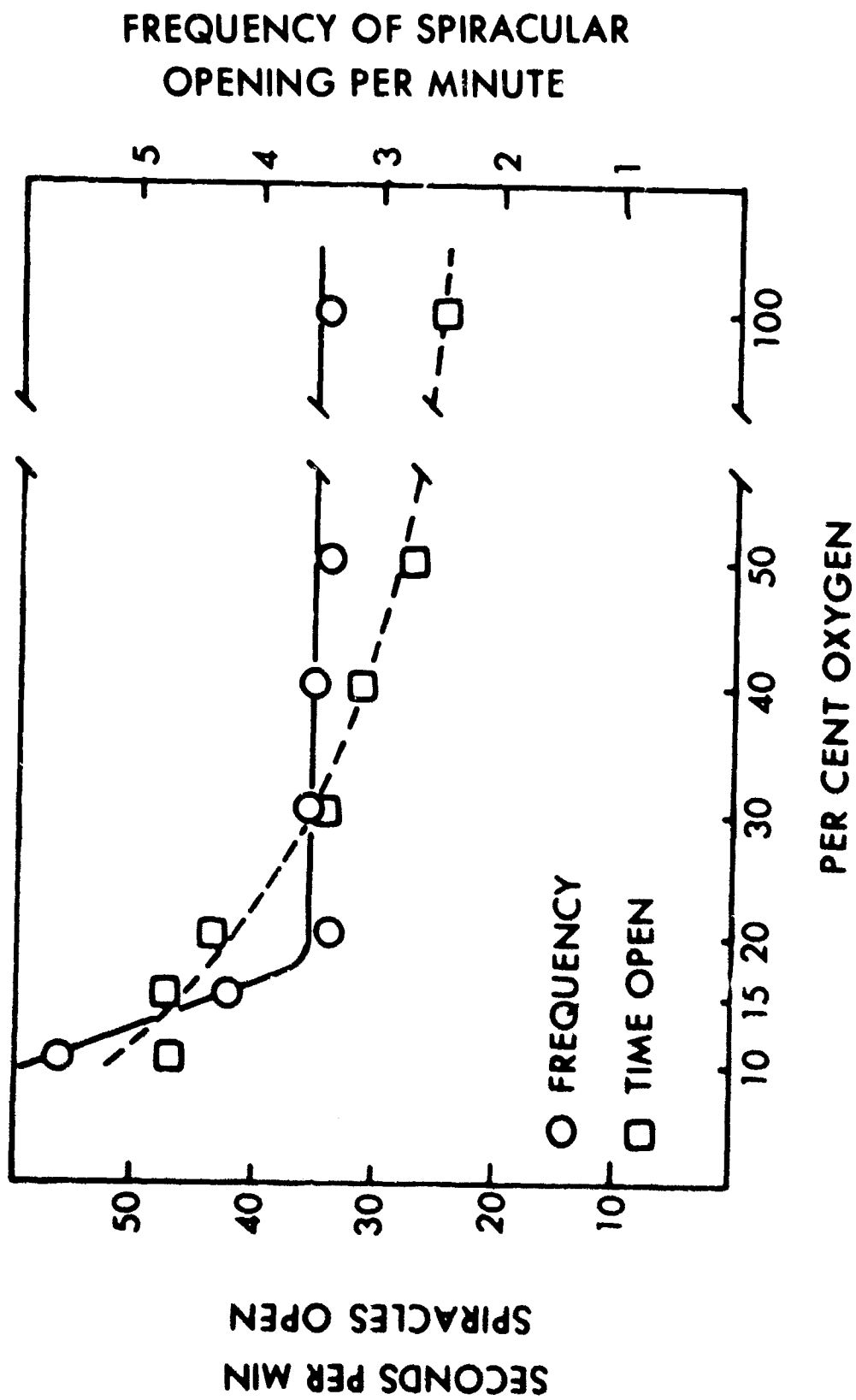


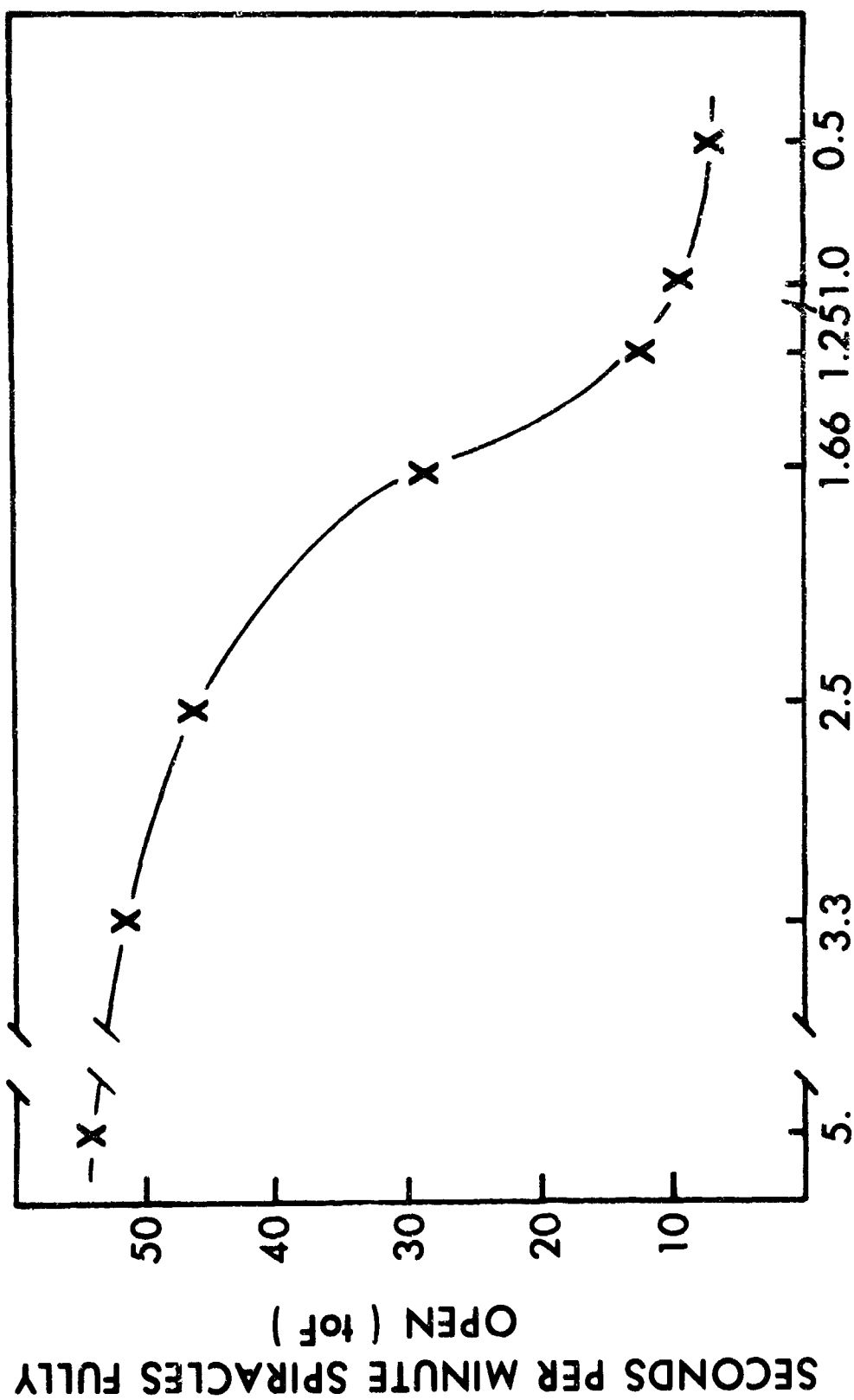
FIGURE 1. Frequency of Spiracular Opening and Total Time of Opening in *A. triseriatus* as Functions of O_2 Concentration. Each point is the average of 30 one-minute observations.

($P < 0.10$) and it may be stated that frequency of spiracular opening (and hence closing) is directly related to the relative concentration of carbon dioxide to oxygen.

TABLE 2. SPIRACULAR RESPONSES IN A. TRISERIATUS
(SAY) TO VARYING LEVELS OF OXYGEN AND NITROGEN
WITH A CONSTANT LEVEL OF CARBON DIOXIDE
AT 0.005% OF THE TOTAL ATMOSPHERE

% O ₂	CO ₂ /O ₂	Freq.	S.E.	toT	S.E.
10%	5.00	1.50	.48	55.6	1.76
15%	3.33	1.70	.33	54.4	1.58
20%	2.50	2.11	.36	52.7	1.62
30%	1.66	3.23	.31	39.3	2.30
40%	1.25	3.68	.34	38.9	1.34
50%	1.00	3.35	.22	31.0	2.46
100%	0.50	3.05	.29	32.1	2.89

Amplitude of spiracular opening was the one feature of spiracular behavior clearly showing a dose-dependent response to the ratio of carbon dioxide to oxygen (Fig. 2). The rate of increase in toF was greatest between 2.5 and 1.25% carbon dioxide of the oxygen concentration, and here the slope amounted to an 11.4% increase in duration of toF per 0.25% increase in ratio of carbon dioxide to oxygen. Beyond these values, rates of change in toF were attenuated. It is therefore apparent, in light of Hassan's¹³ finding that spiracular opening is the result of relaxation of the spiracular closer muscle, that carbon dioxide (i) effects a greater degree of muscle relaxation and (ii) extends the period of relaxation beyond that determined by the normal frequency of motor impulses to the muscle. Support for the latter contention may be found in Table 2, which indicates that an inverse relationship exists between frequency of spiracular opening and proportional time of spiracular opening; the relationship is particularly clear if full spiracular opening (toF) is compared with frequency. It would be interesting to know if, in fact, the spiracular responses to ambient ratios of carbon dioxide to oxygen applied here reflect responses occurring to similar intratracheal gas tensions. This point will be taken up later.



PER CENT CO₂ OF O₂ CONCENTRATION

FIGURE 2. Amplitude of Spiracular Opening in *A. triseriatus* as a Function of the CO₂/O₂ Ratio. Each point is the average of 30 two-minute observations.

In this study only the initial sensory effects of the ratio of carbon dioxide to oxygen on spiracular behavior were measured, because it was necessary to gather an adequate number of samples rather than make extensive observations on a limited number of insects. It was therefore desirable to investigate spiracular response to carbon dioxide as a function of duration of exposure. A limited series of observations were made on six A. triseriatus and six A. aegypti mosquitoes observed first in air for 1 minute and subsequently in air plus 1% carbon dioxide for 20 minutes. Observations were recorded for 2-minute periods with 2-minute intervals between. Five such periods of observation were made over a 20-minute period for each mosquito. The results (Fig. 3) clearly indicate that spiracular response to carbon dioxide became attenuated with increasing duration of exposure; thus, spiracular behavior in the presence of ambient carbon dioxide progressively approaches that observed in normal atmospheres. Similar responses were obtained by Case² in Callitroga and Musca exposed to several carbon dioxide tensions.

In a second series of observations, five each of A. aegypti and A. triseriatus were exposed to 8% oxygen in nitrogen after first being observed in air. Responses to lower than "normal" oxygen tension, like that to carbon dioxide, lessened with increasing duration of exposure (Fig. 4). It therefore seems that accommodation results upon extended exposure to other than normal carbon dioxide and oxygen tensions.

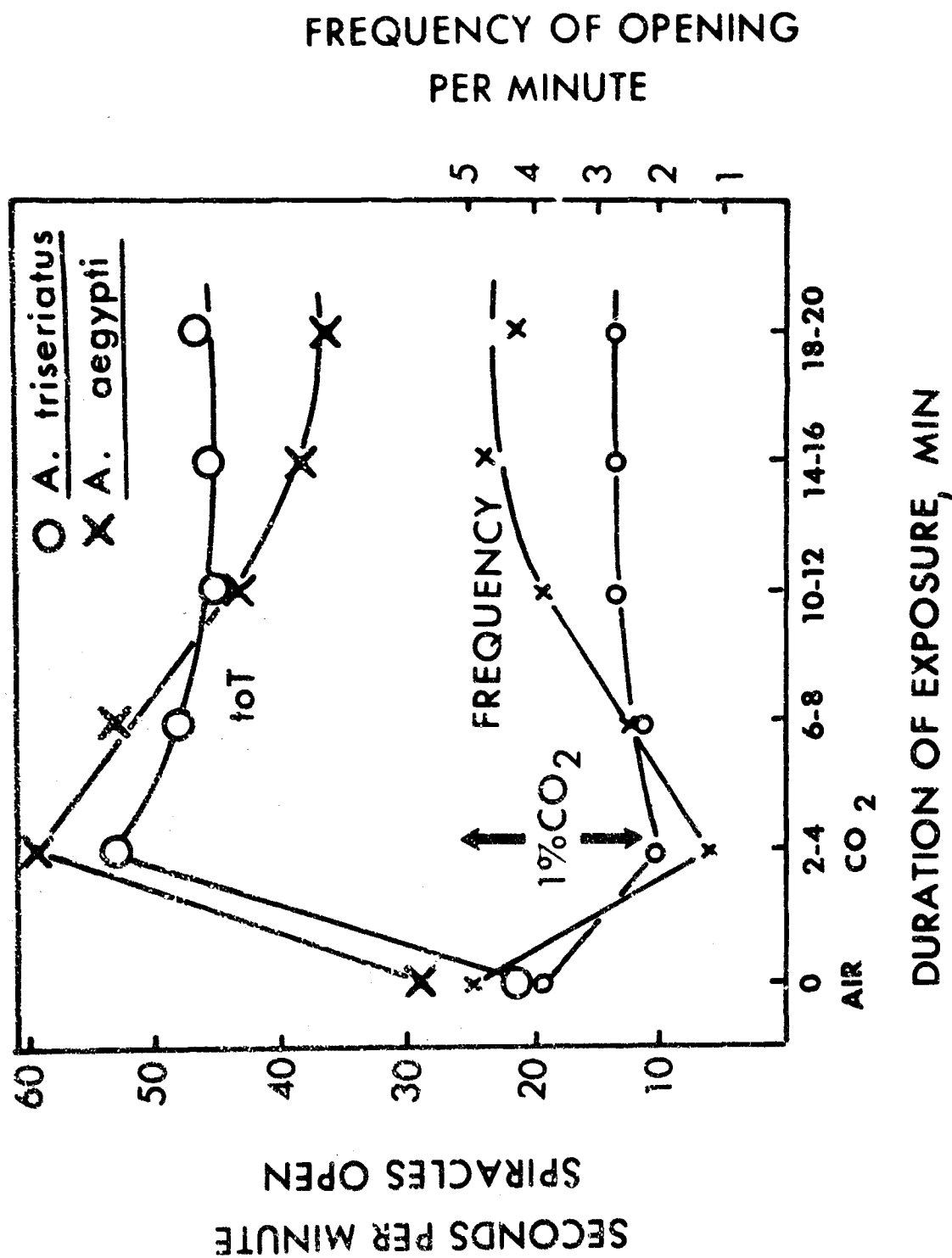


FIGURE 3. Spiracular Responses of A. aegypti and A. triseriatus to 1% Carbon Dioxide in Air as a Function of Duration of Exposure. Each point represents six observations.

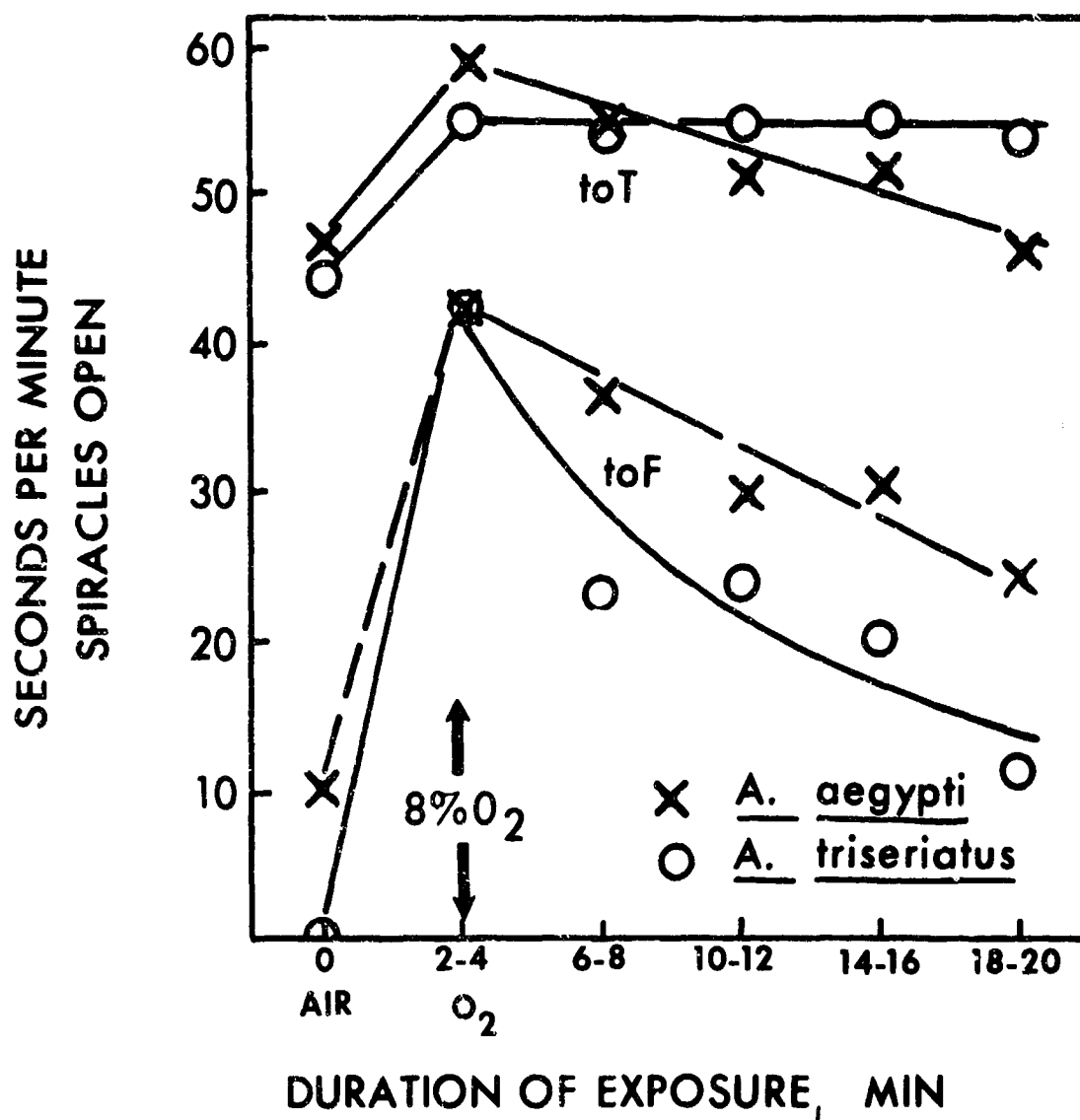


FIGURE 4. Spiracular Responses of A. aegypti and A. triseriatus to 8% Oxygen as a Function of Duration of Exposure. Each point represents five observations.

IV. DISCUSSION

The extraordinary sensitivity of Aedes spiracles to rather low tensions of carbon dioxide is in contrast to the amounts required to elicit spiracular opening in other insects. In houseflies about 6% carbon dioxide was necessary for full spiracular opening; in Schistocerca 5% failed to elicit a complete spiracular response under most conditions.⁵ In diapausing saturniid pupae, concentrations greater than 5% carbon dioxide were required.⁷ Beyond the ambient and intratracheal oxygen tension, the critical concentration of carbon dioxide necessary to elicit opening of the spiracle has been shown to vary with diet,¹¹ age and water balance,¹⁴ ionic balance of the insect,^{6,15} and anything affecting the general metabolism. Our observations may indicate only that the respiratory rate of Aedes is simply higher than that of the other forms cited above. There are other possibilities, however far-fetched they may presently seem. Carbon dioxide in very low concentrations has a function in the host-seeking behavior of mosquitoes.¹⁵ Perhaps a reflex action is excited by carbon dioxide, resulting in preflight behavior and a sudden elevation in metabolism. It is interesting that dragonflies possess a central inhibitory reflex responsible for complete spiracular opening during flight.⁹

Aedes thoracic spiracles behaved as those of Xenopsylla in oxygen pressures less than atmospheric; that is, they showed an increased frequency of opening and closing. However, the duration of each opening was comparatively unaffected in Aedes, but in the flea duration was shortened with decreasing oxygen tension. In greater than atmospheric oxygen pressures, the frequency of spiracular opening in Aedes was unaffected but the duration of opening was attenuated; in the flea, frequency of opening was reduced but duration of each opening increased.¹¹ Abnormal oxygen tensions had no predictable effect on frequency of spiracular opening in diapausing saturniid pupae.⁷ It is remarkable that so little effect of oxygen tensions above 40% was observed in Aedes mosquitoes. Clearly, lack of oxygen had an obvious and immediate effect upon spiracular behavior, but the converse was not so apparent.

By providing a constant amount of carbon dioxide and varying the proportions of oxygen and nitrogen, responses were obtained that differed strikingly from those in the same oxygen - nitrogen mixtures alone. Furthermore, spiracular behavior was clearly related to the ratio of carbon dioxide to oxygen in that the more carbon dioxide relative to oxygen pressure, the greater the reduction in number of spiracular openings, the greater the duration of opening and, most obvious of all, the greater duration of time that spiracles were fully open. It may be significant that Aedes showed predominately increased duration of complete opening with increasing effective carbon dioxide tension (Fig. 1) but Musca showed increased amplitude of opening, i.e., "graded opening."² Although graded (partial) openings of the spiracles do occur in Aedes, our observations indicated that usually the opening was either narrow, short, and twitch-like, or the complete or nearly complete opening was that previously described.¹² Spiracular opening in Aedes is not an all-or-none phenomenon.

It may be useful to interpret the observations presented here in the light of recent knowledge of the mechanism of spiracular responses to carbon dioxide and oxygen. Relaxation of the spiracle closer muscle with consequent opening of the spiracle was first thought to be the result of an accumulation of acid metabolites formed by metabolically produced carbon dioxide.¹¹ Case³ advanced the view that acids simply forced carbon dioxide from solution in the tissues and hemolymph; consequently, it was the gas in the extracellular environment rather than the acid that caused a spiracular response.

Further efforts disclosed that the isolated spiracular apparatus of Hyalophora pupae spontaneously contracted in the absence of any innervation; further, carbon dioxide effected relaxation in this myogenic muscle and oxygen reduced its sensitivity to carbon dioxide.¹⁸ In the American cockroach¹⁷ and the desert locust Schistocerca^{4,5} the occlusor muscle contracts in response to a steady stream of motor impulses from the central nervous system. Abrupt cessation of motor impulses leads to muscle relaxation and spiracular opening. Carbon dioxide apparently does not affect the frequency of impulses to the muscle, however, for Hoyle⁸ demonstrated that, despite continuous motor nervous excitation, carbon dioxide caused relaxation of the closer muscle. Hoyle's investigations suggested that carbon dioxide acted directly on the neuromuscular junction, effecting a reduction in twitch tension and junctional potential. Thus carbon dioxide reduced the response of the spiracular muscle to central control; this explains the peripheral action of the gas noted in earlier studies. Support for the work cited above was given by Miller.⁹ In four species of adult dragonflies, carbon dioxide had a peripheral and direct effect upon the closer muscle; hypoxia acted via the central nervous system, reducing the frequency of motor impulses to the spiracle and thus heightening its sensitivity to carbon dioxide. In the rather special case of the spiracular apparatus of saturniid pupae, Van der Kloot¹⁰ provided evidence that carbon dioxide hyperpolarized the closer muscle and this slowed or eliminated spontaneous activity. Again, carbon dioxide acted directly upon the muscle and not through the central nervous system.

If the frequency of spiracular opening in Aedes is a reflection of the rather complicated electrical events taking place at the spiracle occlusor muscle, then Miller's contention that hypoxia reduces motor impulse frequency would adequately explain the effects of oxygen at less than atmospheric pressures. Here it may be postulated that the closer muscle is more prone to depolarization, thus bringing about an increase in frequency of opening. Less easy to rationalize, however, are the effects of hyperoxic conditions on frequency of spiracular opening, for here the latter was unaffected. Under hyperoxic conditions it may be expected that metabolically produced carbon dioxide would be physiologically less effective in inducing spiracular opening; this was borne out when a constant pressure of carbon dioxide was maintained in the face of increasing oxygen tensions.

Royle's⁵ proposal that carbon dioxide acts directly upon neuromuscular events in causing spiracular opening is consistent with the data presented here. Even in the lowest effective concentrations (i.e., in relation to the oxygen pressure) this gas exerted immediate reductions in the frequency of opening and closing.

In Musca and Callitroga, the reaction time of single spiracles to carbon dioxide gas was directly proportional to its concentration.³ The oriental rat flea, judging by Wigglesworth's figures, gave similar reactions. In our series of observations no systematic records were kept of this, but it can be stated that even in the lowest effective carbon dioxide tensions, response times were essentially immediate, although exceptions did occur. In particular, if a spiracle was in the toD position, application of carbon dioxide effected immediate opening; if the spiracle were closed, the effect of carbon dioxide was often not apparent until the next rhythmical opening was due.

The responses of Aedes spiracles to ambient carbon dioxide and to hypoxia were reduced with increasing duration of exposure. Similar results were obtained in the housefly² with regard to carbon dioxide. If the effect of hypoxia in Aedes is to increase sensitivity to metabolically produced carbon dioxide, then our observations may indicate that accommodation to low oxygen is in reality accommodation to carbon dioxide. It has been suggested that the attenuated response seen in Musca is adaptation rather than fatigue.² Desiccation was invoked to explain similar phenomena in tsetse flies,¹⁴ but whatever the mechanism involved, accommodation to sudden increases in carbon dioxide pressure would surely act as a brake on spiracular transpiration of water.

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